REMARKS

Applicants have considered the outstanding official action. It is respectfully submitted that the claims are directed to patentable subject matter and are in condition for allowance as set forth below.

Restriction is required under 35 U.S.C. §121 and §372 as between Group I, claims 1-6, drawn to a device for characterizing spheroids, and Group II, claims 7-10, drawn to a method for characterizing spheroids. Applicants confirm the earlier election to prosecute in the present application the claims of Group I, i.e., claims 1-6, drawn to a device for characterizing spheroids. Claims 7-10 have been canceled. However, applicants reserve the right to file a divisional application on the non-elected subject matter of Group II, i.e., claims 7-10, drawn to a method of characterizing spheroids, under the provisions of 35 U.S.C. §121.

Applicants are submitting herewith a substitute specification as required.

Applicants have amended the abstract of disclosure to be one paragraph as required. Accordingly, withdrawal of the objection is respectfully requested.

The disclosure is objected to because of informalities that (1) the section heading "Brief

Description of the Drawings" is missing and (2) the word

"resultss" (page 4, line 41 of original specification) is

misspelled. Applicants have added the appropriate

subheadings to the substitute specification and have

corrected the misspelling. Withdrawal of the objections to

the disclosure is respectfully requested.

The claims are objected to due to line spacing.

The informality has been corrected. Withdrawal of the

objection to the claims is respectfully requested.

Claims 4-6 are objected to under 37 CFR §1.75(c)

as being in improper multiple dependent form. Applicants

have amended the claims as to form to correct the improper

multiple dependencies. Withdrawal of the objection to the

claims is respectfully requested.

Claim 1 is rejected under 35 U.S.C. §112, second

paragraph, as being vague and indefinite based on the phrase

"its" and the phrase "to-be-characterized". Claim 4 is

rejected under 35 U.S.C. §112, second paragraph, as being

vague and indefinite based on the phrase "said region (1),

said tube (1,2) has an inner diameter of between 0.1 and 0.5

mm". Claim 6 is rejected under 35 U.S.C. §112, second

paragraph, as being vague and indefinite based on the phrase

"step-like". Claims 1, 4 and 6 have been amended to

overcome the rejections. It is submitted that claims 1, 4

and 6 are definite. Withdrawal of the §112 rejections is respectfully requested.

Claims 1-6 are rejected under 35 U.S.C. §103(a) as being unpatentable over "Rheology of Blood Cells in Sickle Cell Disease" (Damay) in view of U.S. Patent No. 5,686,309 (Frank) and "Four-Electrode Null Techniques for Impedance Measurement with High Resolution" (Schwan). Claim 1 is the sole independent claim. Claim 1 claims a device for characterizing spheroids comprising a tube with a longitudinal axis, the tube containing spheroids and having an inner diameter in a constricted region of the longitudinal axis which is smaller than the diameter of the spheroids. The tube is further claimed as being composed of an electrically insulating material at least at an inner circumference. The claimed device further comprises a first pair of electrodes in the tube on a first side of the constricted region and a second pair of electrodes in the tube on a second side of the constricted region, which lies opposite the first side, with each pair of electrodes having an inner electrode and an outer electrode of which the inner electrode lies closer to the constricted region than the outer electrode. The claimed device further comprises a measurement arrangement having an alternating current source which is connected to the outer electrodes and a voltage meter which is connected to the inner

electrodes, the measuring arrangement producing an impedance spectrogram.

Damay teaches a method of modeling cell deformation by introducing a cell into a tube having an inner diameter in a constricted region that is smaller than the diameter of the cells. In Damay, a fluorescent method is used to model the rheological behavior. Damay does not disclose any electrical arrangement comprising electrodes or any measurement arrangement for performing impedance measurements, in particular for producing an impedance spectrogram. The object of the device of Damay is to deform the cells in order to be able to model the cell deformation.

Contrary to Damay, the object of the claimed invention is not to deform cells. The constricted region of the claimed invention ensures that the spheroids are in mechanical contact over the entire circumference with the electrically insulating inner wall of the tube, so that no electrical bypass occurs and the applied current always flows through the spheroid. This ensures that impedances and impedance spectra of the spheroids can be measured with high sensitivity. The slight deformation of the spheroids necessary for this purpose is very small compared with the deformation necessary for the characterization in the device disclosed in Damay.

Contrary to the statements of the Examiner, Damay does also not disclose a constricted region of a tube in which the inner diameter of the tube changes in steps along the longitudinal axes as claimed. Damay shows in Figure 5.1. an arrangement in which a step is indicated for forming the constricted region. In the constricted region, however, no change of the inner diameter of the tube occurs as claimed.

Damay is applied in combination with Frank and Schwan to reject claims 1-6 under 35 U.S.C. §103(a). Frank teaches a method of characterizing cells by measuring a DC and a RF impedance of the cells in a tube having a constricted region with two electrodes, which are connected to a current source and a voltage source. In the device taught by Frank, however, the inner diameter of the tube in the constricted region is not smaller than the diameter of the red blood cells to be examined. The diameter of red blood cells lies in the range between 6 and 9 μm, whereas the inner diameter in the constricted region (measuring aperture) has dimensions of approximately 50 μm, as can be seen from all of the four examples of Frank. In order to ensure that only one cell passes the measuring aperture at a time, Frank teaches to dilute the red blood cells with a isotonic solution. (See column 6, lines 20-25). Due to this dilution, statistically only one cell passes the

measuring aperture at a time. A deformation of the red

blood cells does not occur due to a mechanical impact of the

tube walls, but due to the fluid forces exerted upon these

cells during flow. (See for example column 11, lines

31-32).

Frank teaches that the DC impedance and RF

impedance are measured when the cells pass the measuring

aperture, deriving corresponding pulse signals. (See column

4, lines 7-26). From these two signals, the hemoglobin

concentration of each of the blood cells can be calculated.

(See column 4, lines 27-30). Frank does not teach measuring

an impedance spectrum and therefore the measurement

arrangement taught by Frank does not produce an impedance

spectrogram as claimed.

Due to the different measurement objects taught by

Damay and Frank (i.e., determining deformation

characteristics in Damay and determining the hemoglobin

concentration of individual red blood cells in Frank), it

can not be obvious for a skilled person in the art to

combine the two references. Since with the impedance

arrangement of Frank the deformation of the cells can not be

measured (Frank proposes other means for measuring such a

deformation in column 9, lines 24-50), a skilled person in

the art would not include in the device taught by Damay an

electronic system as taught by Frank in order to determine

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deformation characteristics. Moreover, Frank does not disclose any measurement arrangement for producing an impedance spectrum which is part of the claimed device.
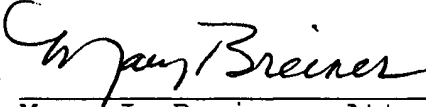
The same applies to a combination with the four electrode arrangement of Schwan which does not give any further motivation for a combination of the above references.

Accordingly, Frank and Schwan do not make up for the shortcomings of Damay as set forth above. Neither Frank nor Schwan suggest any motivation to modify the teachings of Damay, Frank or Schwan in order to provide the claimed invention. Accordingly, Damay in combination with Frank and Schwan does not render the claimed invention obvious within the meaning of 35 U.S.C. §103(a). Withdrawal of the §103 rejection is respectfully requested.

Reconsideration and allowance of the claims are respectfully requested.

Respectfully submitted,

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Attachment - Substitute Specification

DEVICE AND METHOD FOR CHARACTERIZING SPHEROIDS

The present invention relates to a device and a method for characterizing cell structures aggregated under microgravitation conditions. Under microgravitation conditions aggregated 3D cell structures, so-called spheroids, can be employed as models for manners of proceeding in gene technology and pharmacology.

Using spheroids as models for manners of proceeding in pharmacology and gene technology requires characterizing them with regard to the effect of drugs respectively of gene manipulations.

Presently molecular-biological methods, such as for example nucleinic acid hybridization or utilization of antibodies are employed for characterizing. Evaluation can occur by means of fluorescence microscopy. For this, however, complicated slides have to be prepared.

This method of characterizing spheroids is therefore complicated and requires experienced skilled staff for evaluation. A high throughput, desirable particularly in industrial application, automation and nondestructive characterizing are not possible with the prior art methods.

The object of the present invention is to provide a device and a method for characterizing spheroids, which

permits a high throughput, automation as well as

nondestructive characterization of spheroids.

The object is solved using the device and the

method according to claim 1 respectively claim 7.

Advantageous embodiments of the method and the device are

the subject matter of the subclaims.

The invented method and the invented device is

based on characterizing spheroids by using impedance

spectroscopy.

Hitherto bio-impedance measuring was employed to

characterize and monitor tissue damage and organ damage, for

skin studies as well as tumor research and dental research.

For example, electrodes were brought directly into contact

with the tissue. Impedance spectrograms of cultivated cell

structures were made, in that the cell structures were

cultivated on planar electrode substrates and the impedance

between the electrodes was determined or in that the cell

cultures were cultivated on filter membranes and the

impedance was determined through the cell layer and the

filter membrane (cf. e.g. J. Wegener et al., J. Biochem.

Methods 32 (1996), 151-170). Proceeding in this manner is

not possible with spheroids.

In the invented method, the spheroids are

introduced into a tube having a smaller inner diameter than

the diameter of the to-be-characterized spheroids in at least one region of their longitudinal axis, referred to hereinafter as positioning region. In this positioning region, the tube is composed either completely of an electrically insulating material or is provided with electrically insulating properties on its inner circumference, for example due to a coating with an insulating layer.

The tube, for example a capillary, is first filled with a culture medium free of any air bubbles. Then the spheroid is introduced into the narrow positioning region of the tube in such a manner that, due to the smaller inner diameter of the tube, it is in mechanical contact with the inner walls of the tube over the entire circumference. Then a current flow is generated along the tube axis over the culture medium and the spheroid by the introduced electrodes and the voltage drop over the spheroids is measured. The impedance is formed by the current and the voltage. In order to produce an impedance spectrogram, the impedance of the spheroid is usually determined over a coherent frequency region.

A relationship that can be utilized for characterization can be produced between the impedance spectrogram and the build up of spheroids respectively its

change, for example, in the region of the cell membrane, the cytoplasm or the intracellular space.

In the invented method, impedance spectroscopy of spheroids is permitted, in particular, by the spheroid having mechanical contact over the entire circumference with the electrically insulating inner wall of the tube so that no current can flow past the spheroid over the culture medium or other paths when feeding in the current, which would lead to faulty measuring resultss. Due to this arrangement, the current always flows through the spheroid. Thus, impedances and impedance spectra of spheroids can be measured with high sensitivity. In this manner, the rapid and nondestructive characterizing of these spheroids is possible. In particular, parameters for automatic test systems can also be gained from the impedance spectra so that testing the effect of drugs and genetic manipulations can be realized on spheroids with a high throughput.

The invented arrangement consists of the tube is composed of an electrically insulating material or coated with a corresponding coating — at least in the positioning region — and has in the positioning region, where the spheroid is positioned during measuring, an inner diameter that is smaller than the diameter of the to-be-characterized spheroid. A first pair of electrodes having an inner and an

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outer electrode is disposed on one side of this region.
Disposed on the second side of the positioning region
located opposite in direction of the longitudinal axis of
the tube, is a second pair of electrodes having an inner and
an outer electrode. In each case, the inner electrode lies
closer to the positioning region than the outer electrode.
The electrodes can be placed at the inner circumference of
the tube or can extend along the inner volume of the tube.

Furthermore, the device is provided with a
measuring arrangement for feeding in an alternating current
between the two outer electrodes and for determining the
resulting alternating voltage between the two inner
electrodes. Of course, all the electrodes have to be
contactable from outside the tube. The measuring
arrangement can, for example, consist of a commercially
obtainable impedance analyzer.

The invented device permits rapid and
nondestructive characterization of spheroids. Due to the
arrangement with the smaller small tube diameter for
positioning the spheroids and the pairs of electrodes
disposed on both sides in the longitudinal direction of the
tube, the shunt paths have very high resistance and, due to
the arrangement of the separated electrodes, the influence
of electrode polarization is negligible for generating the

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current flow and measuring the voltage. It is particularly

due to this that the impedance of the spheroids, which

usually have low resistance, can be determined with high

sensitivity.

Of course, carrying out the measurement, the

diameter of the tube has to be adapted to the diameter of

the spheroids — or inversely, because too small spheroids

would not be in contact with the inner wall of the tube over

the entire circumference. The size of the spheroids lies

usually in the range between 0.1 and 0.5mm so that the

diameter of the tube has to lie in the same range.

Preferably a plurality of tubes of different

diameters are at disposal for characterizing spheroids of

different sizes. The individual spheroids can, for example,

be preselected according to size by means of a perforated

screen, which ensures a reproducible measurement in which

the spheroids are always pressed into the tube to the same

degree.

Preferably the tube has a conical-shaped

enlargement on one or both sides of the positing region

permitting simple and rapid introduction of the spheroids

into the positioning region without any damage. The

electrodes are preferably disposed in the conical—shaped

enlarged region and extend radially into the tube. Due to

this enlargement in this region, the electrodes do not hinder introduction of the spheroids.

For positioning the spheroids in the tube, the spheroids are preferably pressed into or drawn into the tube via a pump acting on the culture medium. Control of the correct position can occur by optical means.

In a preferred embodiment, a current flow, however, is generated by the electrodes during the positioning procedure and the resulting resistance is measured. If the spheroids are positioned correctly, this resistance increases markedly. This control can, for example, occur by means of measuring the direct current resistance.

Of course, the tube can also be designed conical-shaped in the positioning region so that it is possible to characterize spheroids having different diameters which attach themselves at different points of the conical-shaped positioning region. However, the reproducibility problem arises here, because the degree of compression of the spheroids and, thus, their length respectively their resistance along the tube axis depends on the pressure force. This problem can be avoided with a constant tube diameter.

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In another preferred embodiment, a tube is provided in which the inner diameter changes in the positioning region in steps along the longitudinal axis. Spheroids of different sizes can also be attached by this means.

The invented device and the corresponding method permit measuring a spheroid in a very short time. Measurement of the impedance can be conducted in less than 1 second. Positioning time lies in the range of a few minutes or less.

Especially for industrial use, an array-like arrangement of a multiplicity of invented devices is advantageous, when, for example, they have different diameters. A multiplicity of spheroids can be characterized in parallel by this means. Furthermore, the use of tubes with a constant cross section over the positioning region permits introduction of the spheroids from one side of the tube and expulsion of the spheroids after measuring on the opposite side of the tube so that continuous throughput can be achieved in automatic measuring systems.

A preferred field of application of the present method respectively of the corresponding device is in the field of (chemo) therapeutic testing (pharmacology, pharmakinetics; side effects) and their effect mechanisms.

For example, proof of gene-therapeutic approaches on cancer tumor spheroids can be conducted with it. With the aid of impedance spectroscopy using the present method respectively device with a positioned gene-manipulated tumor cell spheroid permits determining morphological changes, disintegration of the tissue and an increase in necrotic areas from the impedance changes in the cell membrane in the shortest time in a reproducible manner. Thus, use of the present device offers a rapid and efficient method of proving the effectiveness of gene constructs for use in tumor gene therapy.

The present invention is described once more in the following using preferred embodiments with reference to the accompanying drawings, showing in:

Fig. 1: a cross section of a detail of a preferred embodiment of an invented device with a positioned spheroid;

Fig. 2: a diagrammatic representation of a preferred embodiment of the invented device for characterizing spheroids by means of impedance spectroscopy; and

Fig. 3a/b: a cross section of two further examples of the geometric shape of the tube of the invented device.

In this preferred embodiment, the invented device
consists of a tube having an inner diameter of 0.2mm in the
positioning region of the spheroid and an inner diameter of
4mm outside this positioning region. Such a type of tube,
as shown in figure 1, can be produced from a narrow
capillary 1 of insulated material, as for e.g. glass to both
ends of which, glass tubes 2 having a larger diameter are
fused.

In this example, the capillaries have a length of
8mm and the small glass tubes a length of 40mm. The
transition of the inner diameter of the small glass tubes 2
and the glass capillaries runs conical-shaped.

In the two fused-on small glass tubes 2, on both
sides of the positioing regions, a first borehole is
provided at a distance of 15 mm from the center of this
region and a second borehole at a distance of 20m from the
center of this region respectively. The boreholes have a
diameter of 0.4mm. Four platinum wires 3,4 with a length of
10cm and a diameter of 0.3mm are glued into the boreholes.

The platinum wires form the outer electrodes 4
respectively the inner electrodes 3 for receiving the
impedance spectogram. The given distances of the electrodes
from the entrance of the tube are, of course, only intended
as an example and have no significant influence on the

measurement. The electrodes may also be disposed in the tube in another manner, for example, as a coating.

Moreover, figure 1 shows the culture medium 5 filled into the tube without any air bubbles and the positioned spheroid 6 pressed into the positioning region. For conducting the measurement, an alternating current is applied to the two external electrodes 4. The drop in alternating voltage over the spheroid is detected by the two inner electrodes 3.

Figure 2 depicts a diagram of an example of the entire invented device. This figure shows the narrow positioning region of the glass capillary 1, the two outer glass tubes 2 having a large inner diameter and the outer electrodes 4 and inner electrodes 3. In order to introduce the spheroid, the glass body 1,2 is attached with the electrodes at a holding means. The lower opening of the glass tube is connected by means of a flexible tube 7 filled with the culture medium 5, in this case having a length of 20cm and an inner diameter of 5mm, to a fine-control valve 8 having a pressure-release valve.

The culture medium is pressed from the flexible tube into the glass body 1,2 via the fine-control valve until the glass body is completely filled with the culture medium 5. Then the to-be-characterized spheroid 6 is

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introduced into the culture medium through the upper opening

of the glass body. After this a flexible tube 9, which is

filled with oil, is connected to the upper opening of the

glass body. The flexible tube filled with oil is connected

with its other end to a moveable piston 10. Then the

pressure-release valve 8 is opened. If the spheroid 6 has

sunk due to the gravitational force in the conical-shaped

transition from the glass tube 2 to the capillary 1, the

spheroid is centrally positioned by suited operation of the

moveable piston 10. The central position of the spheroid is

shown in the figure. In order to position the spheroid,

differences in pressure are generated in the capillary by

means of the moveable piston via the oil-filled flexible

tube. After positioning, the spheroid 6 remains in the

corresponding position into which it was pressed.

The four platinum electrodes 3, 4 are connected to

an impedance analyzer, consisting of a current source 11 and

a voltage meter 12. Via the outer electrode 4s, a current

$i = I^* \times \sin(\omega t + \varphi_1)$ is fed in, which is regulated in such a

manner that the drop in voltage $u = U^* \times \sin(\omega t + \varphi_u)$ over

the inner electrodes 3 is approximately 10 mV. This

alternating voltage is detected by means of the voltage

meter 12. The impedance analyzer derives the amount and the

phase of the impedance from the current and the voltage. In

order to obtain the characteristic impedance spectrum of the spheroid 6, the impedances are determined over the frequency range of 20 Hz to 1 Mhz.

Figures 3a and 3b show two further examples of the geometric shape of the tube of the invented device. The tube cross section changes steplike over the positioning region 1. In addition, the embodiment of figure 3b is provided with bulges which prevent the spheroid 6 from changing position if, for example, light forces unintentionally act on the spheroid via the liquid surrounding the spheroid.

Both the embodiments permit taking up spheroids 6 of various sizes, as the figures distinctly indicate. Of course, always only one spheroid is placed in the tube during measuring. The three spheroids 6 shown in the figures are depicted simultaneously only for illustration. Preselection of the spheroids according to size is not necessary if the tube has this shape. Control of the correct positioning can occur, for example optically or electrically as previously explained herein.